

MicroRNA-892b influences proliferation, migration and invasion of bladder cancer cells by mediating the p19^{ARF}/cyclin D1/CDK6 and Sp-1/MMP-9 pathways

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Abstract. Cancers often utilize microRNAs to suppress tumor suppressor genes, thus facilitating their potential for growth and invasion. In the present study, we report the novel findings that miR-892b inhibits proliferation, migration and invasion of bladder cancer cells. The basal expression level of miR-892b was significantly lower in 3 different bladder cancer cell lines than in normal human urothelial cells. Transfection of miR-892b mimics to bladder cancer cells resulted in dose-dependent growth arrest. Flow cytometric analysis of the cell cycle showed that miR-892b-transfected bladder cancer cells were subject to arrest in the G1 phase, which was due to the down-regulation of cyclin D1 and CDK6 followed by upregulation of p19^{ARF}. In addition, overexpression of miR-892b impeded the migration and invasion of EJ cells. Expression of MMP-9 in EJ cells was blocked by transfection of miR-892b; the effect was regulated, at least in part, by activation of the Sp-1 transcription factor. Overall, we verified that miR-892b regulates the p19^{ARF}/cyclin D1/CDK6 and Sp-1/MMP-9 signaling networks in bladder cancer cells and may provide a treatment option for advanced-stage bladder cancers.

Introduction

Bladder cancer is the fifth most common cancer, with a lifetime incidence rate of 1 in 26 for men in the US. The American Cancer Society stated that ~74,000 new cases were diagnosed in the year 2015; of these cases, ~16,000 were expected to die from the disease (1). The incidence of bladder cancer in males is 4 times higher than it is in females, and it is 2 times higher

in white people than in black people for unknown reasons (1). The initiation and progression of bladder cancer is affected by various risk factors such as genetic alterations, and exposure to chemicals and carcinogens in cigarette smoke (1,2). Pathologically, transitional cell carcinoma (TCC) makes up ~90% of cases, and the other 10% consists of neoplastic lesions including squamous cell carcinoma, adenocarcinoma, sarcoma and small cell carcinoma. Although early detection followed by surgical resection extends the chances of survival, the 5-year survival rate for patients with advanced-stage bladder cancer is significantly less than 10% (1).

Bladder cells are transformed by a multistep process which includes genetic alterations such as chromosomal abnormalities (3), loss of tumor suppressor genes (4,5) and amplification of oncoproteins (6). Loss of tumor suppressor genes such as p53 or Rb is common in early stages, while amplification of oncoproteins is more common in advanced stages. However, the particular mutations that give rise to bladder cancer are not yet clearly understood, and even the mutational status of tumor suppressors is unclear. Despite its high impact on public health, there have been relatively few studies on the mechanisms of tumorigenesis of bladder cancer compared to other types of cancers. Therefore, it is critically important to identify molecular targets in order to find novel therapeutic options for this deadly disease.

MicroRNAs (miRs) are small non-coding RNA fragments that regulate gene expression by binding to the 3'-untranslated regions (3'-UTRs) of their target genes (7). When normal bladder epithelia are transformed, as evidenced by alterations in protein expression, dysregulation of miRs is also observed. Previous studies showed that miRs act as either oncogenes or tumor suppressors in bladder cancer. Oncogenic miRs are mainly upregulated (8-10) and tumor-suppressive miRs are downregulated (11-13). Similarly, our previous studies demonstrated that miR-20b-induced p21^{WAF1}-mediated G1 phase cell cycle arrest of bladder cancer EJ cells, suggesting that the miR has a tumor suppressive function (14). In the present study, we reported that miR-892b inhibits proliferation, migration and invasion of bladder cancer EJ cells by regulating the p19^{ARF}/cyclin D1/CDK6 and Sp-1/MMP-9 cascade.

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Materials and methods

Materials. Polyclonal antibodies against cyclin E, CDK2, CDK6, cyclin D1, p53, p19ARF, p21WAF1, p27KIP1 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP-9 polyclonal antibody was obtained from Chemicon International (Billerica, MA, USA). miR-892b (5'-CACUGGCUCCUUUCUGGGUAGA-3') and miR-892b inhibitor were designed and synthesized by Genolution Pharmaceuticals, Inc. (Seoul, Korea).

Cell cultures. Human bladder carcinoma cell lines (EJ, 5637 and T24) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with glucose supplemented with 10% fetal calf serum, L-glutamine and antibiotics (Biological Industries, Beit Haemek, Israel) at 37°C in a 5% CO₂ humidified incubator. Normal human urothelial cells (HUCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were maintained in urothelial cell medium with supplements according to the manufacturer's protocol.

Quantitative real-time RT-PCR (qRT-PCR). Quantification of miRNA expression was performed using a Rotor-Gene™ 6000 as previously described (15). Real-time PCR assays were carried out using the miScript PCR Starter kit (Qiagen Korea, Seoul, Korea). The PCR reaction was performed in a final volume of 20 µl (10 µl 2X QuantiTect SYBR-Green PCR Master Mix, 2 µl 10X miScript Universal Primer (U6), 2 µl 10 pmol forward primer, 2 µl template cDNA and RNase-free water). Real-time PCR conditions were as follows: 1 cycle of initial activation for 15 min at 95°C, followed by 50 cycles of 15 sec at 94°C for denaturation, annealing for 30 sec at 55°C and extension for 30 sec at 70°C. The melting program was performed at 70-99°C at a heating rate of 1°C/5 sec. Spectral data were captured and determined using Rotor-Gene™ Real-Time Analysis Software 6.0, Build 14. All experiments were carried out in triplicate. U6 was used as a control to normalize the quantity of miRNA.

Bioinformatics analysis. Screening for possible targets of miR-892b was performed with the miRanda algorithm (<http://www.microrna.org/microrna/home.do>) and the NCBI mRNA database (NCBI mRNA DB: <http://www.ncbi.nlm.nih.gov/>).

Cell proliferation. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (16). Cell morphology was photographed using phase-contrast microscopy.

Transfection. Cells were transfected with miR-892b and the miR-892b inhibitor using Lipofectamine 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol.

Flow cytometric cell cycle analysis. Cells were harvested and fixed in 70% ethanol. After being washed with ice-cold phosphate-buffered saline (PBS), cells were incubated with

RNase (1 mg/ml) followed by propidium iodide (50 mg/ml). The phase distribution of the cell cycle was determined by a Becton-Dickinson FACStar flow cytometer equipped with Becton-Dickinson Cell Fit software.

Immunoblot analysis. Preparation and quantitation of protein lysates were performed as previously described (14). Lysates were electrophoresed on a 10% polyacrylamide gel (SDS-PAGE) under denaturing conditions and transferred to nitrocellulose membranes (Hybond; Amersham Corp., Arlington Heights, IL, USA). The blots were blocked with 5% (wt/vol) non-fat dry milk in Tris-buffered saline (TBS) [10 mM Tris-HCl (pH 8.0), 150 mM NaCl], and then, the membranes were incubated with primary antibodies at 4°C overnight. The blots were then incubated with peroxidase-conjugated secondary antibodies for 90 min. A chemiluminescence reagent kit (Amersham Corp.) was utilized for the detection of the western blot analyses. The experiments were repeated at least 3 times.

Immunoprecipitation and immune complex kinase assays. Cell lysates were prepared using ice-cold lysis buffer as previously described (14). Briefly, after centrifugation of the lysates at 10,000 x g for 5 min, the supernatants were precipitated by protein A sepharose beads pre-coated with the indicated antibodies at 4°C for 2 h. The immunoprecipitated proteins on the beads were washed 4 times with 1 ml of lysis buffer and twice with a kinase buffer (14). Finally, pellets were re-suspended in 25 µl of kinase buffer containing 1 µg glutathione S-transferase (GST)-pRb C-terminal (pRb amino acids 769-921) fusion protein (Santa Cruz Biotechnology), 20 µM/l ATP and 5 µCi of [³²P]-ATP (4,500 µCi/mmol; ICN). Subsequently, re-suspended pellets were incubated at 30°C for 20 min with occasional mixing. The kinase reactions were terminated by the addition of 25 µl of 2X Laemmli sample buffer and were heated at 100°C for 5 min. Samples were resolved on 10% SDS-polyacrylamide gels, which were then dried. Radioactive bands were visualized. The migration of GST-pRb was determined using Coomassie blue staining.

Wound-healing migration assay. Cells (3x10⁵) were seeded into 6-well plates in 2 ml medium and were grown to 90% confluency. A clear area was created with a 2 mm pipette tip. After 3 washes with PBS, the plates were incubated at 37°C in serum-free medium. Migration of cells into the clear area was analyzed and photographed using an inverted microscope (magnification, x40).

Invasion assay. Invasion assays were performed using an invasion assay kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions. Cells (2.5x10⁴) were re-suspended in serum-free medium and plated in the upper chamber. Media with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells in the lower chamber were stained and photographed. Cell invasion was evaluated using a commercial cell invasion assay kit (Chemicon International).

Gelatin zymography. Culture supernatants were resolved on a polyacrylamide gel containing 1 mg/ml gelatin. Gels

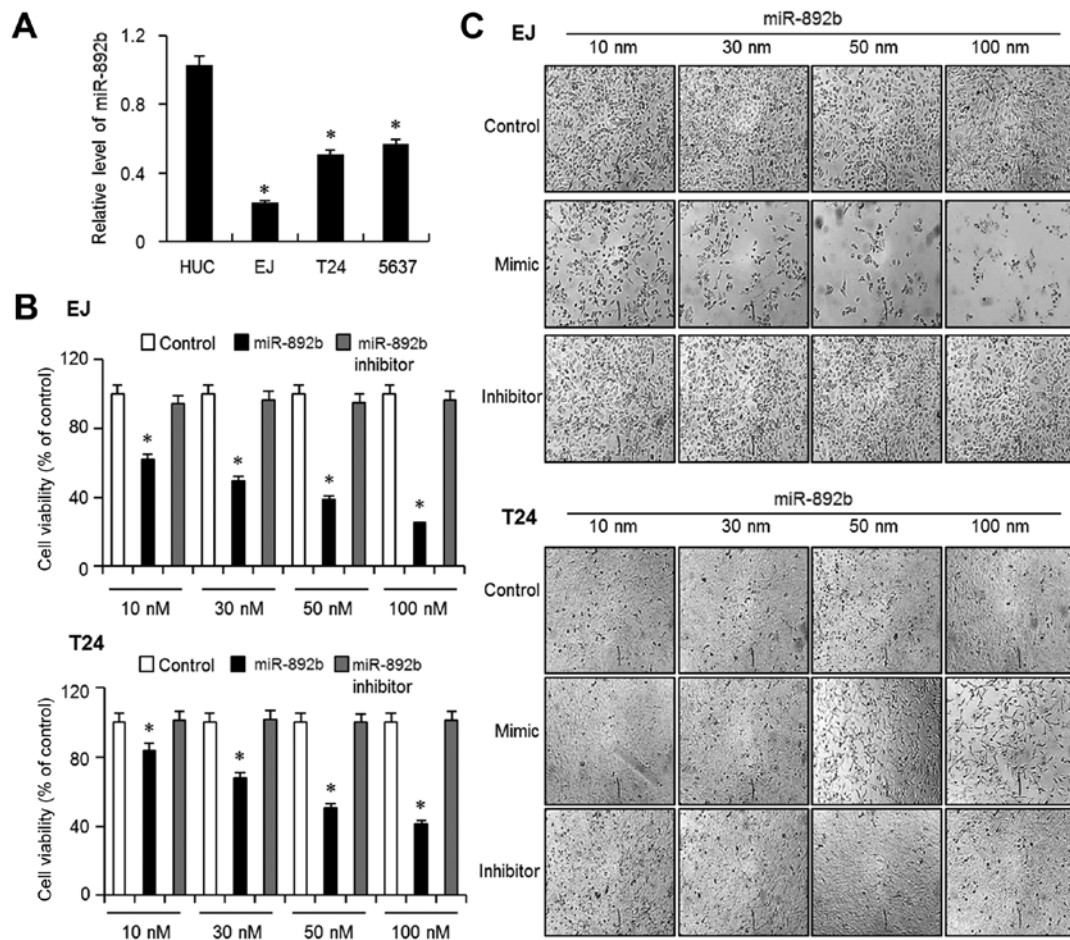


Figure 1. miR-892b expression is decreased in bladder cancer cells, and transfection of miR-892b suppresses the proliferation of bladder cancer cells. (A) The expression of miR-892b was investigated in normal human urothelial cells (HUC) and 3 bladder cancer cell lines (EJ, T-24 and 5637) using quantitative Real-time PCR analysis. The expression of miR-892b was normalized to RNA control (U6) expression. Results are expressed as the mean \pm SE of triplicate experiments; * P <0.01 compared with HUCs. (B) Dose-dependent decrease in proliferation of EJ and T24 cells transfected with miR-892b. Both cell types were transfected with the indicated dose of a miR-892b mimic (miR-892b), miR-892b inhibitor and Lipofectamine 2000 only (control). Forty-eight hours after transfection, cell proliferation was measured using an MTT assay. Results are expressed as the mean \pm SE of triplicate experiments; * P <0.01 compared with the control. (C) Cellular morphology of miR-892b transfectants, miR-892b inhibitor transfectants and controls was captured by phase contrast images.

were washed with 2.5% Triton X-100 at room temperature for 2 h followed by incubation at 37°C overnight in a buffer containing 10 mM CaCl₂, 150 mM NaCl and 50 mM Tris-HCl, pH 7.5. The gel was stained with 0.2% Coomassie blue and photographed with a light box. Areas of gelatinase activity were visible as clear bands in a dark blue field.

Preparation of nuclear extracts and EMSA. Nuclear proteins were extracted as previously described (17). Briefly, cells were washed, scraped and suspended in a buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The cells were lysed with 0.5% NP40. The homogenates were then centrifuged, and the nuclear pellets were extracted with an ice-cold high-salt buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF). After centrifugation, the supernatants containing the nuclear extracts were prepared. Protein concentrations were measured using Bradford reagent (Bio-Rad). An electrophoretic mobility shift assay (EMSA) was performed as previously described (17). In brief, the oligonucleotides spanning the MMP-9 *cis* element of interest were end-labeled with ³²P-ATP by T4 polynucleo-

tide kinase (Promega, Madison, WI, USA). Nuclear extracts (10–20 μ g) were incubated with a radio-labeled oligonucleotide probe (10,000 cpm) at 4°C for 20 min in a binding buffer solution (25 mM HEPES buffer pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol, and 2 μ g poly dI/dC). The DNA-protein complex was resolved at 4°C on a 6% polyacrylamide gel containing a TBE running buffer (89 mM Tris, 89 mM boric acid and 1 mM EDTA). The gel was rinsed, dried and then exposed to X-ray film for 10 h. The sequences for the oligonucleotides were as follows: AP-1, CTGACCCCTGAGT CAGCACTT; NF- κ B, CAGTGGAAATCCCCAGCC; and Sp-1, GCCCATTCCTTCCGCCCCAGATGAAGCAG.

Statistical analysis. Where appropriate, data are expressed as mean \pm SE. Data were evaluated by factorial ANOVA and a Fisher's least significant difference test where appropriate. Statistical significance was set at P <0.05.

Results

Basal level of miR-892b expression is low in bladder cancer cells. The aim of the present study was to verify the role of

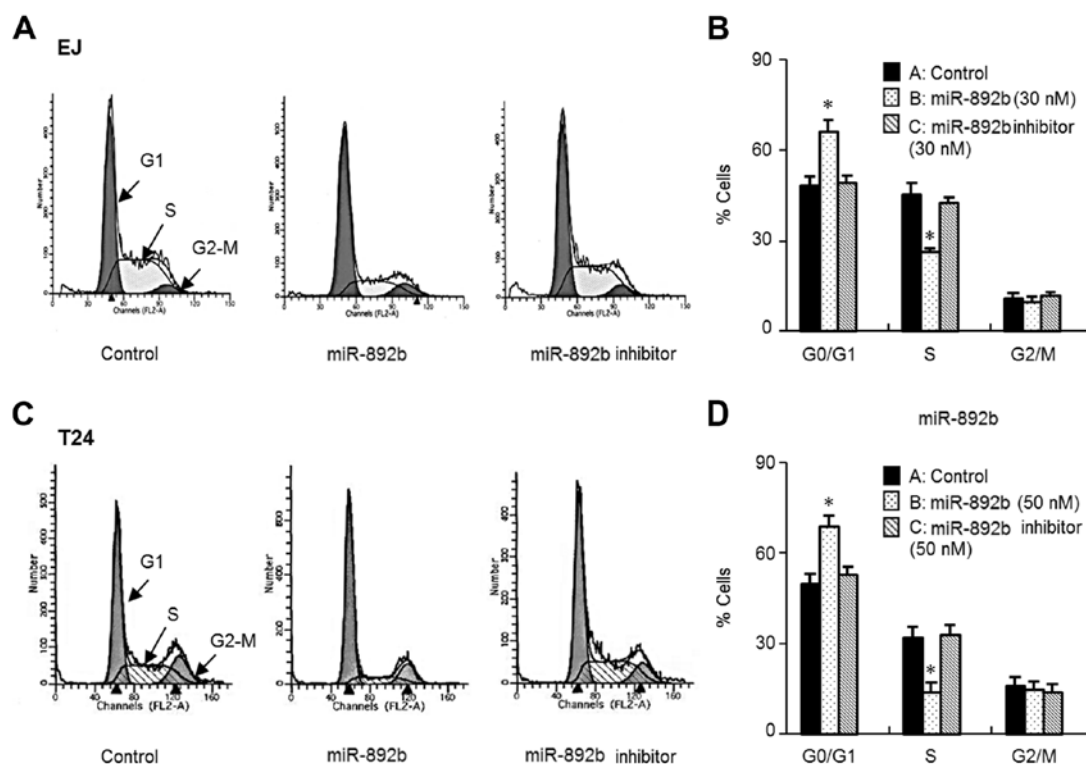


Figure 2. Transfection of miR-892b mimic induces G1 phase cell cycle arrest in EJ and T24 cells. (A and C) Forty-eight hours after post-transfection, flow cytometric analysis was performed to determine the effect of miR-892b on the cell cycle. (B and D) The percentage of cells in each phase of the cell cycle is displayed as the mean \pm SE of triplicate experiments; * $P < 0.01$ compared with the control.

miR-892b in bladder cancer. We first measured the basal expression level of miR-892b in 3 different bladder cancer cell lines, EJ, T-24 and 5637, and one normal human urothelial cell line (HUC). Quantitative real-time-PCR showed that the basal levels of miR-892b were significantly lower in all the 3 bladder cancer cells than in HUC cells (Fig. 1A). EJ and T24 cells exhibited the least expression of the 3 cancer cells; therefore, we selected EJ and T24 cells for further experiments. We then investigated the effect of miR-892b on the proliferation of EJ and T24 cells, and the cells were transfected with Lipofectamine 2000 (control), miR-892b mimic (miR-892b), and inhibitor of miR-892b (inhibitor) at concentrations from 10 to 100 nM. Introducing miR-892b mimic into EJ cells significantly inhibited proliferation in a dose-dependent manner (Fig. 1B). In contrast, neither control nor inhibitor showed any meaningful decrease in the growth of the cells. Images of the transfected cells supported the result (Fig. 1C). Similar results were obtained from T24 cells (Fig. 1B and C). Subsequent experiments were performed with a miR-892b concentration of 30 nM ($\sim IC_{50}$) for EJ cells and 50 nM ($\sim IC_{50}$) for T24 cells, respectively.

miR-892b causes G1 phase cell cycle arrest in bladder cancer cells.

To elucidate the cause of miR-892b-mediated growth inhibition of bladder cancer cells, we investigated the cell cycle phase distribution of miR-892b-transfected cells through flow cytometric analysis. As demonstrated by the DNA histograms of the cell cycle, introduction of miR-892b resulted in accumulation in the G1 phase (Fig. 2A-D). In agreement with that result, G1 phase accumulation was followed by a corresponding

decrease in the S phase population (Fig. 2A-D). Based on the results of flow cytometry, we searched the microRNA database (miRanda) to find possible targets of miR-892b in terms of cell cycle regulators, including cyclins, CDKs and CDK inhibitors. We identified cyclin D1, CDK6 and p19^{ARF} as possible causes of G1 phase cell cycle arrest in bladder cancer EJ cells. First, we investigated changes in protein expression levels of the G1 phase cell cycle regulators in miR-892b transfectants using immunoblots. In line with bioinformatics analysis, the expression of cyclin D1 and CDK6 were significantly reduced in miR-892b transfectants (Fig. 3A). However, the expression levels of cyclin E and CDK2 were not significantly altered. Progression of the cell cycle often requires activation of CDKs, which form a complex with corresponding cyclins. CDK6 couples with cyclin D1 and phosphorylates Rb (18). We investigated the kinase activity of CDK6 in miR-892b transfectants with immunoprecipitation assays. In accordance with the immunoblot results (Fig. 3A), the kinase activity of CDK6 was reduced by $\sim 50\%$ in miR-892b transfectants compared to the control or the miR-892b inhibitor (Fig. 3C). This strongly suggests that miR-892b targets cyclin D1-associated growth signals in bladder cancer cells.

miR-892b leads to the upregulation of p19^{ARF}, resulting in growth inhibition of bladder cancer cells. Based on the finding that miR-892b-induced G1 phase cell cycle arrest in bladder cancer EJ cells, we investigated the effect of miR-892b on protein levels of CDK inhibitors associated with G1 to S phase cell cycle progression. Immunoblot analysis demonstrated upregulation of p19^{ARF} in miR-892b-transfected

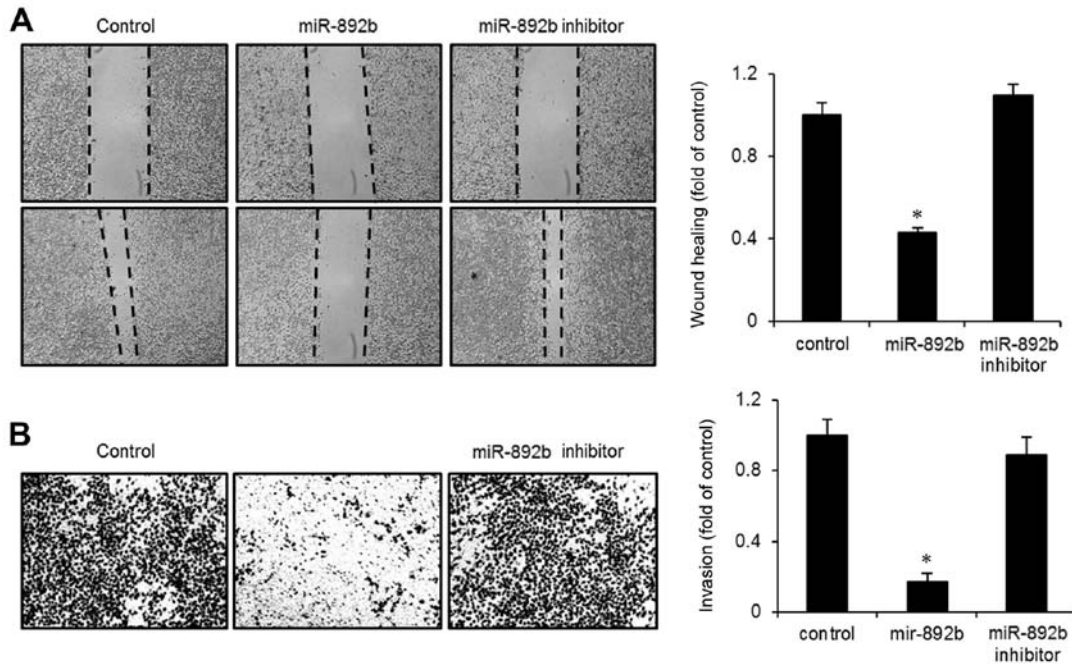


Figure 4. Transfection of miR-892b inhibits the migration and invasion of EJ cells. (A) After transfection of miR-892b, wound-closure rates were compared with those of cells transfected with the miR-892b inhibitor or the control. (B) Invasion of EJ cells was measured by Boyden chamber assays. All results are expressed as the mean \pm SE of triplicate experiments; *P<0.01 compared with the control.

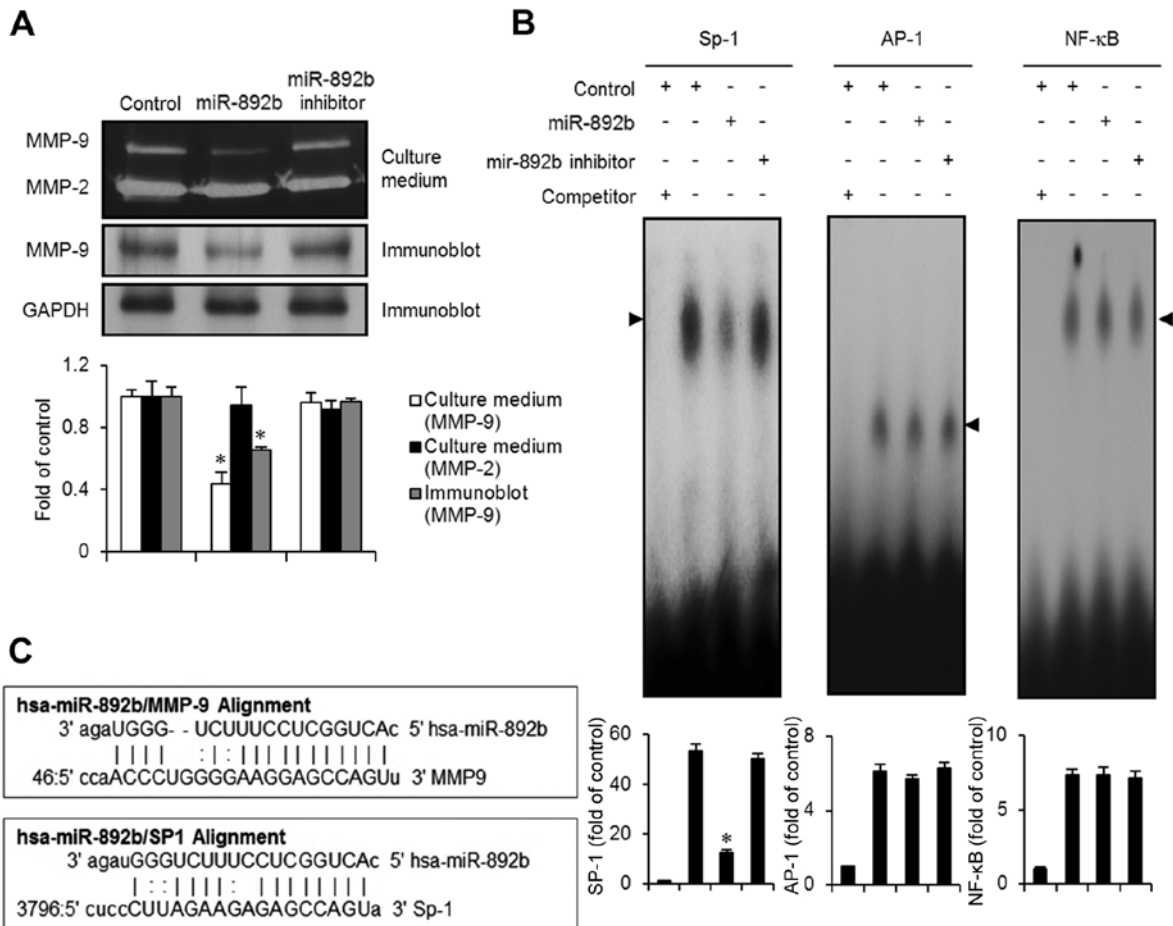


Figure 5. Transfection of miR-892b downregulates MMP-9 expression via suppression of Sp-1 binding activity in EJ cells. (A) Activity of MMP-9 in miR-892b-transfected EJ cells was measured by gelatin zymography. (B) EMSA was performed to assess the change in the binding activity of transcription factors in miR-892b transfectants. For the competition (competitor) assay, unlabeled Sp-1, AP-1 or NF- κ B oligonucleotides were added. (C) Sequence alignment of the 3'-UTR of MMP-9 and Sp-1 targeted by miR-892b. All results are expressed as the mean \pm SE of triplicate experiments; *P<0.01 compared with the control.

showed a reduction in MMP-9 expression (Fig. 5A). miRanda bioinformatics analysis for microRNA targets identified Sp-1 as one of the targets of miR-892b. Thus, using EMSA assays, we investigated whether transcription factors including Sp-1, AP-1 and NF- κ B are modulated by miR-892b transfection in EJ cancer cells. Nuclear extracts from miR-892b transfectants exhibited a reduction in activity of the Sp-1 binding motif (Fig. 5B). However, no specific binding activity of AP-1 or NF- κ B was observed in the transfectants (Fig. 5B). Taken together, these results suggest that the Sp-1 transcription factor is involved in the suppression of MMP-9-mediated migration and invasion of bladder cancer EJ cells induced by miR-892b.

Discussion

Bladder cancer is one of the most common types of cancers worldwide, causing annually ~150,000 deaths (21). Standard therapeutic options to date for advanced bladder cancer consist of cisplatin-based combinatorial chemotherapies, although new therapeutic approaches are being explored. Earlier studies by others demonstrated that microRNAs regulate cellular functions such as cellular proliferation, differentiation and cell death (7,22). miR-892b was previously found to be a modulator of MCL1 in colorectal cancer, sensitizing cells to a BCL inhibitor, ABT-263 (navitoclax) (23). However, the cellular function of miR-892b and its cellular signaling networks in cancer states have not been reported.

Based on predicted-target bioinformatics analysis of miR-892b, we found that miR-892b regulates signaling molecules associated with cellular processes including proliferation, migration and invasion of bladder cancer cells. Upon initial evaluation, the basal expression of miR-892b was shown to be significantly lower in all bladder cancer cell lines tested than in normal human urothelial cells, which indicated that miR-892b may be associated with transformation of bladder cells. As speculated, transfection of a miR-892b mimic to bladder cancer cells resulted in growth arrest. Transfection of a miR-892b inhibitor did not cause any alteration in the growth of bladder cancer cells. These results suggest that, in bladder cancers, a cellular mechanism exists to downregulate miR-892b, which limits its tumor suppressive function. In addition, we found that miR-892b participates in the progression of the cell cycle from G1 to S phase in bladder cancer cells. Among known G1 phase cell cycle regulators, we examined the expression level of cyclin D1, cyclin E, CDK2 and CDK6. Cyclin D and E are key regulators of the cell cycle and are associated with the initiation and progression of bladder cancer (24,25). We found that cyclin D1, but not cyclin E, was downregulated in miR-892b-transfected EJ bladder cancer cells. Further immune complex kinase assays showed that the activity of CDK6, but not that of CDK2, was reduced by miR-892b transfection. Notably, Zhao *et al* reported that targeting CDK6 in bladder cancer cells by transfecting miR-29c reduced the growth and invasiveness of bladder cancer cells (26). In agreement with that result, we found that introduction of miR-892b to bladder cancer cells significantly inhibited proliferation by lowering protein levels of cyclin D1 and CDK6. The activity of CDKs is tightly controlled by CDK inhibitors such as p16^{INK4A}, p19^{ARF}, p21^{WAF1/CIP1} and p27^{KIP1}. Mutations in CDKs are frequently observed in certain

types of cancer, as in the case of p16^{INK4A} in melanoma (27). However, signature mutations or mutation hot spots in CDKs for bladder cancers are relatively understudied. Previously, the correlation of p21^{WAF1/CIP1}, p27^{KIP1} and p53 levels were examined in 51 patient-derived tumor specimens with overall survival rate. They concluded that downregulation of p27^{KIP1} and overexpression of cyclin D1 and D3 predicted survival of bladder cancer patients (28). Another recent study showed that miR-451 inhibits the proliferation of esophageal carcinoma cells by targeting p19^{ARF} (29). In the present study, p19^{ARF} was upregulated in miR-892b transfectants. However, p21^{WAF1/CIP1} and p27^{KIP1} levels remained unchanged. These results demonstrated for the first time that miR-892b impedes proliferation of bladder cancer cells through G1 phase cell cycle arrest by inducing the p19^{ARF}/cyclin D1/CDK6 cascade pathway.

Managing invasiveness of bladder cancer is one of the most difficult clinical challenges. The 5-year survival rate for patients with stage 4 bladder cancer is ~10% for both men and women, which is attributable to invasiveness or metastasis (1). In the present study, we found that miR-892b inhibits the invasion and migration of bladder cancer cells through wound recovery and Boyden chamber invasion assays. During disease progression, bladder cancer cells penetrate surrounding muscle tissues through the generation of proteolytic enzymes such as matrix metalloproteinases (MMPs) (30). Previous studies demonstrated that MMPs are closely correlated with poor prognosis in bladder cancer patients (19,20,31). Similarly, we previously identified that MMP-9, but not MMP-2, was associated with invasion of bladder cancer cells (14,17,33). The activity of MMP-9 in bladder cancer cells is tightly regulated by transcription factors, including Sp-1, AP-1 and NF- κ B (17,32,33). In our bioinformatic analysis, Sp-1 was identified as a binding candidate for miR-892b. Utilizing EMSA assays, we verified that Sp-1 was the critical transcription factor regulated by miR-892b. However, neither AP-1 nor NF- κ B was regulated by miR-892b. Our data suggest that Sp-1 is essential for miR-892b-mediated suppression of MMP-9 expression leading to the reduction in the migration and invasion of bladder cancer cells.

In conclusion, we demonstrated that aberrant expression of miR-892b is associated with progression of bladder cancer cells. Re-introduction of a miR-892b mimic significantly inhibits the proliferation and migration of bladder cancer cells, which suggests that miR-892b may be a novel target for treatment of bladder cancer.

Acknowledgements

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